Alternative chemo-enzymatic treatment for homogeneous and heterogeneous acetylation of wood fibers

Elisabet Quintana[a] (0000-0002-4273-9204), Mariko Ago[b] (0000-0001-5258-4624), Cristina Valls[a*] (0000-0003-2307-1779), M. Blanca Roncero[a] (0000-0002-2694-2368), Orlando J. Rojas[b*] (0000-0003-4036-4020)

[a]*CELBIOTECH_Paper Engineering Research Group. Universitat Politècnica de Catalunya. BarcelonaTech. 08222 – Terrassa. Spain*

[b]*Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, FIN-00076, Espoo, Finland*

*Authors for correspondence: Cristina Valls, E-mail: cristina.valls@upc.edu . Tel.:+34 937 398 190; Fax: +34 937 398 101 and Orlando Rojas, E-mail: orlando.rojas@aalto.fi . Tel.: +358 505 124 227

ABSTRACT

A new chemo-enzymatic treatment is proposed to produce cellulosic fibers suitable for heterogeneous- or homogeneous-phase acetylation. The procedure included enzymatic (laccase-violuric acid) lignin removal from the precursor fibers (unbleached sulfite pulp) followed by hydrogen peroxide treatment. An optional intermediate stage included partial hydrolysis (endoglucanase) to increase fiber reactivity. The obtained "biobleached" fibers were acetylated in the heterogeneous phase with acetic anhydride in nonpolar solvents, yielding various acetyl group contents, depending on the severity of the reaction. The degree of acetylation was highly sensitive to the treatment conditions, mainly the acetic anhydride activity in the system. The results were compared to those obtained after acetylation of commercial, dissolving-grade fibers, used as reference. The effect of the inherent nature of the fibers tested were elucidated as far as hemicellulose content, fiber length, fine content and crystallinity (NMR). Acetyl group content of up to 24% were determined after heterogeneous reaction with the chemoenzymatic fibers. The substitution of hydroxyl groups by acetyl moieties resulted in a lower hydrophilicity, as assessed by measurement of the water contact angle. Homogeneous acetylation of the chemo-enzymatic and reference fibers resulted in relatively similar acetyl group content (up to 36 and 33%, respectively). These samples were soluble in acetone and produced transparent films (via solvent casting), with enhanced dry strength and lower hydrophilicity. Overall, it is concluded that the proposed chemo-enzymatic treatment is a feasible alternative for the production of fibers that are suitable for efficient acetylation.

Keywords: Chemo-enzymatic treatment; endoglucanase; heterogeneous acetylation; homogeneous acetylation; films; hydrophobicity; acetic anhydride.

INTRODUCTION

Acetylation is a common chemical modification in which acetyl groups (CH_3CO^-) react with the surface hydroxyl groups (OH) of cellulose, making its surface less hydrophilic. The acetylation process depends on the fiber accessibility and the susceptibility of OH groups in the crystalline and less crystalline domains of cellulose (Kalia et al. 2014). The greater the accessibility, the easier it is for the reactants to diffuse into the interior of the fibers. The generic methods for acetylation are those in heterogeneous (in fiber dispersions) and homogeneous (in solution) phase. The heterogeneous acetylation process is performed in the presence of a non-solvent, such as toluene, benzene or carbon tetrachloride. The reaction product (cellulose acetate) is insoluble and thereby, this process preserves the morphological structure of the fiber. In contrast, cellulose acetate is dissolved during the homogeneous acetylation and, therefore, it demands solvents capable of deconstructing the crystalline network and interacting with the anhydroglucose units of cellulose. This is usually done by reducing or eliminating inter and intra-molecular hydrogen interactions. In the homogenous phase, cellulose begins to react with acetic anhydride, with the initial reaction occurring mainly in the amorphous regions of the structure. Sulfuric acid is used as a catalyst and it combines with the cellulose, forming sulfate linkages; however, most of these are removed during acetylation via exchange with acetyl groups. It is important that the final cellulose acetate contains only a very small amount of sulfate groups because they affect the properties adversely, especially the color. When acetylation is virtually complete, the product of reaction is viscous and clear. The excess of acetic anhydride is then neutralized by adding aqueous acetic acid, which helps to desulfate the residual sulfate linkages (LaNieve and Richard 2007; Luo et al. 2013)

The extent to which the available hydroxyl groups in the repeating unit of cellulose are substituted, the degree of substitution (DS), does not quite reach the maximum of three units per anhydroglucose unit (as in cellulose triacetate). Cellulose triacetate (DS > 2.8) displays a limited solubility in acetone and is reported for use in a relatively narrower number of commercial applications (Cao et al. 2007). Diacetates with a DS from 2.2 to 2.7 (also named secondary acetates)

70 are the most commonly reported cellulose esters. They are soluble in acetone and
71 other organic solvents (Steinmeier 2004; Fischer et al. 2008; Wan Daud and
72 Djuned 2015), and can be used in applications such as coatings, films, textiles,
73 synthetic polymeric membranes, among others.
74 Following a heterogeneous route, it is possible to obtain more crystalline and
75 less biodegradable cellulose acetates (CA) than those produced through
76 homogeneous routes (Barud et al. 2008). On the other hand, the advantages of
77 acetylation in homogeneous phase include the excellent control of the degree of
78 substitution (DS) and the possibility of a uniform distribution of the functional
79 groups along the polymer chain (Ass et al. 2004).
80 Importantly, CA is usually produced from high quality cellulose fibers,
81 namely, dissolving grades derived from cotton or wood (α-cellulose content of >
82 95%) (Saka and Matsumura 2004; Roselli et al. 2014; Wan Daud and Djuned
83 2015). In the case of cotton sources, issues related to the large land area required
84 for farming and water required for irrigation, result in high economic and
85 environmental burdens. Further, the so-called "cotton gap" motivates a need for
86 more extensive utilization of dissolving-grade fibers derived from wood.
87 According to FAO (2012), dissolving-grade fibers constitute a small share of the
88 global pulp production, but prospective consumer markets indicate that this share
89 will increase in the coming decades. Based on this scenario, new technologies are
90 being suggested as alternative to traditional dissolving pulp production processes.
91 In previous studies (Quintana et al. 2013; Quintana et al. 2015a), the laccase-
92 mediator system was used to bleach sulfite pulp and the conversion to dissolving-
93 grade was achieved by cellulase treatment. According to the results, the obtained
94 chemo-enzymatic dissolving-grade fibers exhibited suitable characteristics for use
95 in the synthesis of cellulose derivatives.
96 In the present work, fibers obtained via chemo-enzymatic treatments of
97 biobleached fibers (termed herein as L_E and L_{CE}) were investigated as far as their
98 suitability to synthesize acetylated cellulose. A bleached commercial dissolving-
99 grade fiber, used as a reference and termed "*Com*", was used for comparison. This
100 study focuses on the surface acetylation reactions, typical of heterogeneous
101 acetylation, while homogeneous acetylation was also carried out for comparison
102 purposes. In terms of surface acetylation, given doses of acetic anhydride (Ac_2O)
103 were tested and the degree of acetylation was evaluated by FTIR spectroscopy.

Paper handsheets were produced from fibers that were acetylated on the surface (heterogeneous reaction) and characterized in terms of contact angle, mechanical strength and surface morphology. Samples obtained by homogeneous acetylation were used to prepare transparent films via solvent casting and characterized in terms of the tensile strength and contact angle. This work, therefore, aims at determining if chemo-enzymatic treatment is a suitable alternative for the synthesis of materials with low hydrophilicity via heterogeneous and homogeneous acetylation.

MATERIALS AND METHODS

Precursor fibers

As starting fiber material, *unbleached* sulfite cellulose fibers were used and obtained as a mixture of 60 % Norway spruce (*Picea abies*) and 40 % Scots pine (*Pinus sylvestris*) (Domsjö Fabriker mill, Sweden). Fiber characteristics included a kappa number of 4.2 ± 0.2, ISO brightness of 61.25 ± 0.6 % and viscosity of 511 ± 11 mL/g. The carbohydrate content, as determined by high-performance liquid chromatography (HPLC), was 88.5 ± 0.3 % glucan, 6.0 ± 1.3 % mannan, 2.4 ± 0.4 % xylan and 0.3 ± 0.2 % rhamnan. As a reference fiber source, a totally chlorine-free (TCF) *bleached* sulfite dissolving-grade pulp was employed. This pulp was obtained from the unbleached fibers (as indicated above). It has an ISO brightness of 91.70 ± 0.15 % and viscosity of 474 ± 1 mL/g. The carbohydrate composition, also determined by HPLC, included 95.1 ± 0.3 % glucan, 2.8 ± 0.2 % mannan, 0.8 ± 0.0 % xylan, 0.2 ± 0.2 % rhamnan, 0.2 ± 0.2 % arabinan, 0.3 ± 0.1 % glucuronic acid and 0.2 ± 0.1 % acetic acid. The bleached fibers were obtained by sulfite digestion followed by chemical bleaching at the Domsjö Fabriker mill (Sweden). These fibers, which are used commercially, are thereafter referred to as *Com*.

Enzyme treatment

A laccase (*Trametes villosa*, TvL) was supplied by Novozymes® (Denmark) with an activity of 746 U/mL and used for *biobleaching* the fibers. The laccase activity was measured as the extent of oxidation of 5 mM 2,20-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical (ε_{436} = 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24 ºC. One activity unit (U)

was defined as the amount of enzyme converting 1 µmol of ABTS per min. Violuric acid (VA), the mediator used for this enzymatic treatment, was purchased from Sigma-Aldrich and used as received. A *hydrolytic* treatment was also applied involving an endoglucanase produced from *Cerrena unicolor* (supplied by Fungal Bioproducts®, Spain). The activity measured as U/g dry enzyme powder of the cellulase preparation was 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity, respectively. The activity was determined in our laboratory using the Somogyi–Nelson method.

For biobleaching, unbleached sulfite fibers were first conditioned at pH 4 adjusted with H_2SO_4, stirred at 2 % solids content for 30 min and washed with de-ionized water in a glass filter funnel. This step was needed to remove contaminants and metals, and also to bring the fiber dispersion to the pH required for the enzymatic treatment. The biobleaching process included a sequence denoted as L_{VA}*(PO)(PO)*, where L_{VA} denotes an enzymatic (laccase) treatment and *PO* the hydrogen peroxide stage assisted with oxygen. The enzymatic stage was carried out with the laccase–violuric acid system in an oxygen pressurized reactor (0.6 MPa) at stirring rate of 30 rpm, using 50 mM sodium tartrate buffer (pH 4) to adjust 5 % (w/w) fiber content, at 50 ºC for 4 h. The enzyme dose was 20 U/g odp (oven dry weight of fibers) of laccase and 1.5 % odp of violuric acid (Quintana et al. 2013). The enzymatic treatment was followed by a chemical bleaching stage involving hydrogen peroxide assisted with oxygen. *PO* was carried out at 5 % (w/w) solids in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm under the following conditions: 3 % odp H_2O_2, 1.5 % odp NaOH, 0.3 % odp DTPA and 0.2 % odp $MgSO_4$, at 90 ºC for 1 h. Treated fibers were washed extensively with deionized water, and then followed with another hydrogen peroxide stage assisted with oxygen. The treatment was performed under same conditions described above but 2.5% odp H_2O_2 and 3 h of reaction were used. The chemical stage was finished by washing the bleached fibers with deionized water.

The resulting *biobleached* fibers (L_{VA}*(PO)(PO)*), denoted here as *L*, for simplicity, were used in two different additional treatments to produce the chemo-enzymatic samples used later for acetylation reactions. One was subjected to enzymatic hydrolysis with an endoglucanase (resulting in fibers that are denoted thereafter as L_E). The other included the application of cold caustic extraction before endoglucanase treatment (resulting in fibers that are denoted thereafter as

L_{CE}). The purpose of introducing an endoglucanase treatment was to improve fiber reactivity. By its side, cold caustic extraction was a purification stage where hemicelluloses were removed and, as a result, fiber quality was improved. Both enzymatic treatments were performed in polyethylene bags that were placed in a laboratory water bath, at 10% solids (w/w) in 0.05 M sodium acetate buffer at pH 5.5 at 55 ºC for 1h and with 12 U/g odp enzyme. The samples were periodically kneaded and the reaction was stopped by washing the fibers with de-ionized water in a porous glass filter funnel of porosity grade 2. The cold caustic extraction was also conducted in a polyethylene bag. The treatment was performed at 10 % (w/ w) solids adjusted with 9 % (w/v) NaOH at 25 ºC for 1 h. Treated fibers were washed with de-ionized water until the filtrate pH was neutral (Quintana et al. 2015a).

L_E, L_{CE}, and Com Fiber Analysis

The commercial dissolving grade and chemo-enzymatic fiber samples (*Com*, L_E and L_{CE}) were characterized in terms of kappa number, brightness and viscosity according to ISO 302:2004, ISO 2470:2009, ISO 5351:2004, respectively. The cellulose reactivity of the fiber samples was determined according to slightly modified version of Fock's method (Fock 1959; Köpcke et al. 2010). This is a micro-scale method simulating the industrial viscose process for manufacturing regenerated cellulose. Prior to analysis, the samples were dried at 50 ºC and conditioned in a climate room at 23 ºC and 50% RH overnight. Carbohydrate composition of treated fibers was determined using high performance liquid chromatography (HPLC). Samples were studied by duplicate using a modified version of TAPPI 249 cm-09 test method. Prior to HPLC analysis, samples were filtered using a 0.45 µm pore size Whatman membrane. Chromatographic analysis was performed using a 1200 Agilent HPLC instrument furnished with a Biorad Aminex HPX-87H ion-exchange column. Concentrations were calculated by interpolation in calibration curves ran from standards of glucose, xylose, rhamnose and arabinose. In order to resolve xylose, mannose and galactose peaks, the hydrolyzed effluents were neutralized with barium carbonate ($BaCO_3$), then were filtered through a membrane of 0.45 µm pore size and then were analyzed with a Biorad Aminex HPX-87P column. The chromatographic determination was performed with the following conditions: mobile phase, 6 mmol/L (acid samples)

or ultrapure water (neutralized samples); flow rate, 0.7 mL/min; column temperature, 60 ºC (acid sample) or 80 ºC (neutralized sample).

^{13}C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ^{13}C. Samples were immersed in deionized water for at least 2h. All measurements were performed at 290 ± 1K. The magic angle spinning (MAS) rate was 4 kHz. The cross-polarization contact time was 1 ms and the recycle delay time 2.5 s. Acquisition time was 98.3 ms and sweep-width was 31.2 kHz. The number of scans was 5100.

Heterogeneous (surface) and homogenous (bulk) acetylation

In the heterogeneous phase acetylation, 2.0 g oven dried pulp (odp) of each type (*Com*, L_E, L_{CE}) was disintegrated and then filtered using a filter paper (Whatman 1) for water removal. The samples were then placed in a glass beaker containing a mixture of 20 mL of acetic acid (99.7% w/w) and 35 mL toluene. The dispersion was stirred for 5 min and 0.2 mL sulfuric acid (95% w/w) was added. Then, a desired amount of acetic anhydride (Ac_2O) was added and the mixture was stirred for 1 h at room temperature. The specific conditions for acetylation reactions and the nomenclature used were as follows: 0.53 g (Lowest), 2.67 g (Low), 5.35 g (Medium) and 10.7 g (High) Ac_2O per gram of dried fiber sample (*Com*, L_E, L_{CE}). The "Lowest" conditions were not applied to the L_{CE} pulp. The reaction was quenched by adding 6 mL of distilled water and ethanol, 3:7 v/v. The mixture was allowed to stand for 20 min and then washed 3 times with methanol and finally with water until neutral pH (Fig.1).

Homogeneous acetylation was performed as reference. For this purpose, 2.5 g odp of respective fiber type (*Com*, L_E, L_{CE}) was disintegrated and then filtered using a filter paper for water removal. Then, 50 mL of acetic acid was added to the sample, stirred 5 min and then filtered. This step was done by duplicate. After filtration, 45 mL of acetic acid and 0.25 mL sulfuric acid was dropped into the sample and stirred for 1 min. Then, 5.35 g Ac_2O/g dried fiber (~12.5 mL Ac_2O) was added and continuously stirred for 30 min at room temperature. The reaction was quenched with the addition of 6.25 mL of distilled water and acetic acid at a ratio of 3:7 v/v, respectively. Finally, cellulose acetate (CA) was obtained by pouring the viscous reaction mixture into distilled water obtaining a continuous droplet and with constant stirring. With precipitation, cellulose acetate was

regenerated. The obtained product was washed with distilled water until neutrality and subsequently dried using a freeze-drying (Fig. 1).

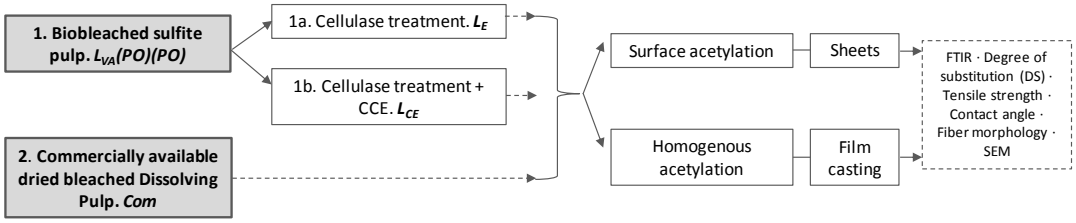


Fig. 1 Outline of experimental procedures and samples studied. Biobleached (*L* or $L_{VA}(PO)(PO)$) sulfite fibers were subjected to cellulase treatment (1a, L_E) or cellulase treatment after cold caustic extraction, *CCE* (1b, L_{CE}). The fibers after L_E, L_{CE} treatment were subjected to heterogeneous (surface) or homogeneous acetylation reactions. Fiber handsheets or films were prepared and characterized. Bleached commercial dissolving fibers (*Com*) were used as a reference, and same heterogeneous and homogeneous acetylation reactions were performed on such reference fibers.

The acetylated samples were analyzed by Fourier transform infrared spectroscopy (FTIR) by using a Nicolet Avatar 360 spectrophotometer (Nicolet Instrument Corporation). The samples were prepared by mixing 1 mg of the sample in a matrix of 300 mg of KBr followed by pressing. The spectrum was recorded in the range of 400– 4000 cm^{-1} and 32 scans were run at 4 cm^{-1} resolution.

Determination of acetyl group content of acetylated cellulose

The nominal degree of substitution was determined according to ASTM D871-96 (2010). Firstly, the respective acetylated sample was ground and 100 mg (oven dried) were weighed accurately and placed into 20 mL of 75% v/v of ethanol in an Erlenmeyer flask. The bottle, loosely stoppered, was heated to 50-60 ºC for 30 min for better swelling of the material. Then, 20 mL of 0.5 N NaOH solution was added to the sample and the mixture was heated to 50-60 ºC for 15 min. A blank was also conducted but in absence of fiber sample. The flasks were stoppered tightly and allowed to stand at room temperature for 72h. The excess alkali was then titrated with 0.5 N HCl using phenolphthalein as indicator. An excess of about 1 mL of 0.5 N HCl was added and allowed the NaOH to diffuse from the regenerated cellulose overnight. The small excess of HCl was titrated with 0.5 N NaOH to a phenolphthalein end point. The percentage of acetyl groups was calculated as follows:

$$Acetyl\ groups, \% = [(D - C)Na + (A - B)Nb] \cdot (F/W)]$$

where *A* and *B* are the volumes (mL) of the NaOH solution (normality = *Nb*) required for titration of the sample and the blank, respectively. *C* and *D* are the volumes (mL) of the HCl solution (normality= *Na*) used for the titration of the sample and the blank, respectively.

F is a constant (4.305) for acetyl and *W* the mass (g) of the sample used.

Handsheets from surface acetylated fibers and cellulose acetate films

Fibers obtained by surface acetylation were used for preparing handsheets. For sheet manufacture 1g of each sample at 1% solids was disintegrated and poured into an over-pressurized device (< 1 bar pressure difference) allowing few minutes drainage to obtain a web or handsheet of the acetylated fibers. The device was equipped with open mesh fabric screen (Sefar Nitex 03-10/2, mesh opening of 10 µm with open area of 2 %) to remove the excess water and retain the fibrils. The webs were pressed between two blotting papers using a metal roller (10 kg) and then dried at 80 ºC, for 1 h in a tumble drier. The obtained sheets were then stored in a conditioned room (23 ºC and 50 % relative humidity) until further use.

Surface acetylated handsheets were used to determine different properties. The morphological characteristics of fibers (viz., length, width and curl), and fine content were determined in accordance with TAPPI T 271 on a Metso kajaaniFS300 fiber analyzer. High-resolution imaging of surfaces (handsheets were taken on a JEOL JSM- 6400 scanning electron microscope (SEM). Samples were placed on the SEM sample holding stub with the aid of conductive double side sticky carbon film and coated with Au/Pd alloy prior to analysis. The wetting characteristics of the acetylated handsheets was determined by the initial water contact angle (WCA) using a Dataphysics OCA15EC contact angle goniophotometer (Dataphysics, USA). A 4 µL water drop was dropped to the sample surface, and an image capture ratio of 25 frames/s was used to calculate the initial contact angle. A minimum of ten readings were taken on every sample to reduce possible influence of the heterogeneity of the surface. Also, changes in contact angle were monitored until complete absorption of each water drop. Wet and dry tensile strength of the surface acetylated sheets were measured on a MTS

297 400/M Vertical Tensile Tester equipped with a 50 N load cell, in accordance with
298 ISO 1924-3:2005.
299 Cellulose acetate (CA) obtained from homogeneous acetylation reaction was
300 used for preparing transparent films by means of a casting technique. Dried
301 cellulose acetate was dissolved in given amounts of acetone in order to obtain a
302 concentration of 8 wt%. The solutions for film casting were firstly centrifuged at
303 6000 rpm for 10 minutes. The supernatant was carefully transferred and
304 centrifuged again at 2000 rpm for 5 minutes. The films were cast by pouring the
305 transparent solution on a glass plates, well distributed and followed by drying in a
306 vacuum desiccator for at least 2h. The film samples were finally kept in a
307 desiccator. Tensile strength tests for CA films resulted from homogeneous
308 acetylation reactions were performed on a MTS 400/M vertical Tensile Tester,
309 with a cross-head speed of 40 mm/min. Specimen strips presented 10 mm width
310 and 40 cm length. Note that comparison of the handsheet (paper) and film samples
311 is not possible since they are quite different systems. The water contact angle,
312 water drop test (Tappi standard T835 om-08) and dry zero-span strength (ISO
313 15361:2000) were also determined.

314 # RESULTS AND DISCUSSION

315 ## Precursor fiber characterization

316 The effect of acetylation on the quality of the systems obtained from L_E, L_{CE}
317 or *Com* was evaluated. The characteristics of respective treated fibers are
318 indicated in Table 1. All applied sequences resulted in similar lignin content, as
319 assessed by the kappa number, ISO brightness and viscosity. However, the
320 commercial dissolving fibers (*Com*) exhibited the highest ISO brightness. Similar
321 values of Fock solubility were found for all the fibers. Some authors obtained a
322 higher Fock solubility if endoglucanases were applied after a cold caustic
323 extraction stage (concentration > 8 wt). This was due to the transformation of
324 cellulose I into cellulose II and the fact that endoglucanases have a greater affinity
325 for the latter allomorph (Engström et al. 2006; Köpcke et al. 2008; Gehmayr and
326 Sixta 2011, Quintana et al. 2015b). However, although L_{CE} solubility tended to be
327 higher compared to L_E, no significant differences were produced. It is also known

that lower viscosity can influence cellulose solubility (i.e. reactivity); however, in general, all fibers presented comparable viscosity.

Table 1 Main characteristics (mean ± standard deviation) of L_E, L_{CE} fibers as well as *Com* reference

	L_E	L_{CE}	*Com*
Kappa Number	< 0.5 ± 0	< 0.5 ± 0	<0.5
ISO Brightness (%)	84.6 ± 0.9	83.7 ± 1.5	90.3 ± 0.1
Viscosity (mL/g)	473 ± 55	447 ± 18	476 ± 1
Fock solubility (%)	66.9 ± 2.9	71.5 ± 2.3	67.3 ± 2.1

The carbohydrate composition was determined by HPLC, with special attention to the hemicelluloses content (Fig. 2). The endoglucanase treatment applied to the biobleached fibers (*L*) to obtain L_E reduced the amount of hemicelluloses by 35%, especially the mannan and galactan fractions. The introduction of a cold caustic extraction followed by hydrolytic treatment (L_{CE}) further decreased the amount of hemicelluloses by 46.2%. To be precise, compared to L_E, L_{CE} treatment contribution amounted to 11.2%, resulting in a smaller xylan fraction and similar mannan and galactan content. The lowest hemicellulose content was measured in *Com*.

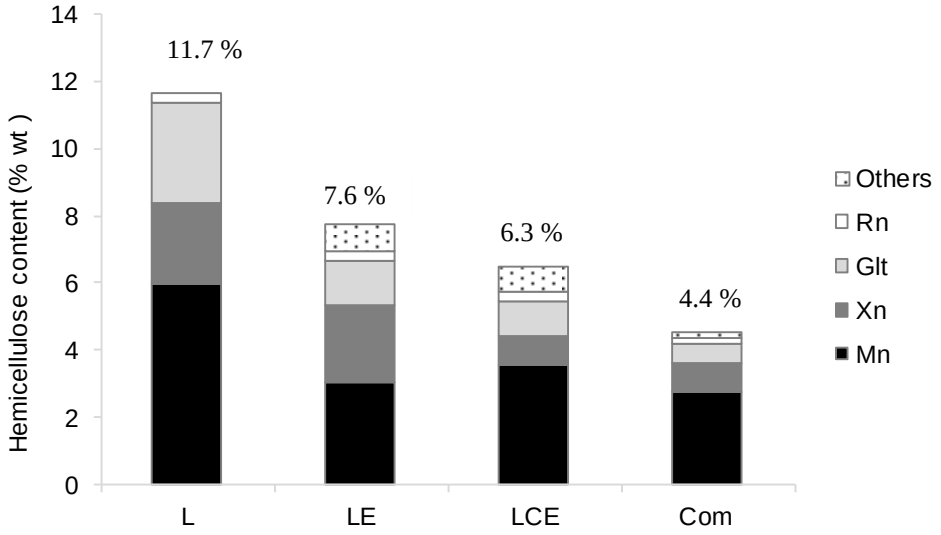


Fig. 2 Hemicellulose composition of biobleached fibers before (*L*) and after chemo-enzymatic treatment *(L_E and L_{CE})*. The composition of commercial dissolving fibers (*Com*) is also indicated.

The total content of hemicelluloses is indicated on top of each column. Mn: mannan: Xn: xylan; Glt: galactan; Rn: rhamnan; Others: it includes acetic acid, glucuronic acid and galacturonic acid

Solid state ^{13}C-NMR spectra of biobleached fibers (*L*), biobleached fibers followed by endoglucanase treatment (L_E), and biobleached fibers submitted to cold caustic extraction (9 % (w/v) NaOH) followed by endoglucanase treatment (L_{CE}) are included as *Supporting Information* (Fig. S1). L_{CE} treatment presented slightly different polymorphic form from unbleached and L_E sample. Caustic extraction converted cellulose I to cellulose II: the C-6 signal at 64 ppm increased, obtaining two peaks with nearly identical heights at 66 and 64 ppm. However, a shoulder at 108 ppm of C-1 signal, which is characteristic of cellulose II, was not observed (Janzon et al. 2008a). Note that the small proportion of cellulose II in L_{CE} is associated with the similar Fock solubility values between samples (Krässig 1993; Janzon et al. 2008b).

The enzymatically-treated pulps displayed properties comparable to those of comercial dissolving pulp. The environmental advantages of enzymatic technologies have been reported previously via Life Cycle Assessments (LCA), which indicated a reduced contribution to global warming. In addition, a reduced contribution to acidification, eutrophication, photochemical ozone formation and energy were noted (Jegannathan and Nielsen 2013). Skals et al. 2008 also reported that the introduction of xylanase in biobleaching contributed to reduce global warming. Zhi Fu et al. 2005 showed that the introduction of a laccase-mediator stage in biobleaching reduced the contribution to ozone depletion and acidification, as well as reducing solid waste generation and energy consumption. Related work highlighted the benefits of producing the enzyme and mediator at the point of use.

Heterogeneous acetylation

Different degrees of surface acetylation were achieved by varying the concentration of acetic anhydride (Ac_2O) in the nonpolar solvent used. The Ac_2O loading correspond to relative activities denoted thereafter as “lowest”, “low”, “medium” and “high”. The effect of acetylation on L_E, L_{CE} and *Com* samples was assesed via FTIR spectroscopy (Fig. 3). Changes in non-acetylated and acetylated samples were identified. Specifically, the structural changes of acetylated fibers

were confirmed by the appereance of three new bands characteristic of the acetyl group vibration at about 1735-1740, 1368-1375 and 1259-1277 cm^{-1}. The peaks located at 1735-1740 cm^{-1} were attributed to the C=O stretching of carbonyl in the ester bonds. The peaks located at 1368-1375 cm^{-1} were assigned to C-H symmetrical deformation in methyl group. The vibration peaks between 1259 and 1277 cm^{-1} corresponded to C-O stretching of the acetyl group. The absence of peaks in the 1840-1760 cm^{-1} region demonstrated that there was no residual, unreacted acetic anhydride in the acetylated fibers (Rodionova et al. 2011; Cunha et al. 2014; Muhammad Djuned et al. 2014; Mashkour et al. 2015).

In the case of *Com* fibers, it is noted that by increasing the amount of Ac_2O used for acetylation resulted in a higher intensity of the C=O band at 1735 cm^{-1}; at the same time, a decrease in the C-O band at 1235 cm^{-1} was clear. Although the C-H band at 1375 cm^{-1} is charactersitic of acetylated fibers, no variation in absorption was evident for the different acetylated conditions. A similar observation applies to L_E and L_{CE} fibers but differences in the intensity peak at 1735 cm^{-1} with respect to the acetylation conditions were less pronounced. For both, L_E and L_{CE}, a high intensity peak at 1735 cm^{-1} was observed when a high (10.7 g) Ac_2O level was introduced.

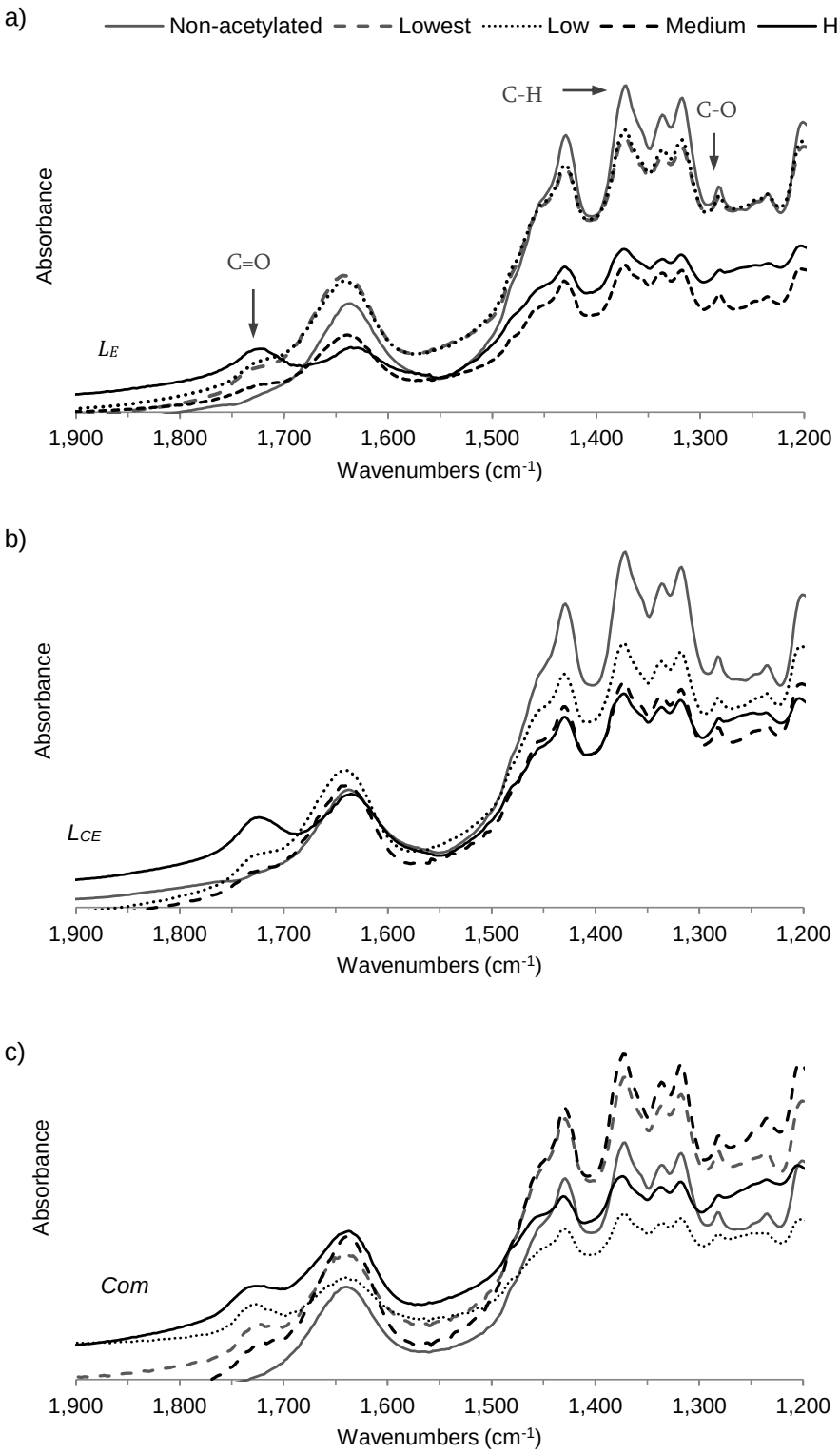


Fig. 3 FTIR spectra for L_E, L_{CE} and *Com* (commercial dissolving fibers) samples at different acetylation levels (lowest, low, medium and high).

The degree of acetylation (i.e. acetyl group content) was determined by titration with NaOH and HCl (Fig. 4). L_E sample did not show differences in terms of acetyl content after reaction with lowest and medium Ac_2O levels, but a

significant gain in acetyl group content was observed for the high dose level (10.7 g). In fact, from all studied fibers, the highest acetyl group content (~24%) was determined for the *LE* sample. In general, compared to *LE*, acetylation of *LCE* fibers yielded a smaller amount of acetyl groups. The application of "low" Ac_2O levels was not effective in incorporating enough acetyl groups. Only by using two or four-fold the Ac_2O dosage level introduced a suitable amount of acetyl groups. Specifically, 13% and 15% of acetyl group content were measured for medium and high Ac_2O dosages. In the case of *Com* sample, a gradual improvement in acetyl group content was observed from the lowest (0.53 g Ac_2O) to the medium (5.35 g Ac_2O) Ac_2O addition. Unexpectedly, a high Ac_2O addition produced a relatively small acetylation degree. *Com* subjected to medium conditions resulted in 15% acetyl group substitution, while only 7.9% was measured at high Ac_2O levels (a 47.3% reduction). These results were not in agreement with FTIR data that indicated that the sample treated under "high" conditions displayed the highest peak intensity in the C=O band. Actually, the same acetyl content was found using low and high amounts of Ac_2O. Several reasons can explain these observations. For example, the distribution of the functional groups along the polymer chain may not be uniform after heterogeneous acetylation, which introduces artifacts in the determination of acetyl group content.

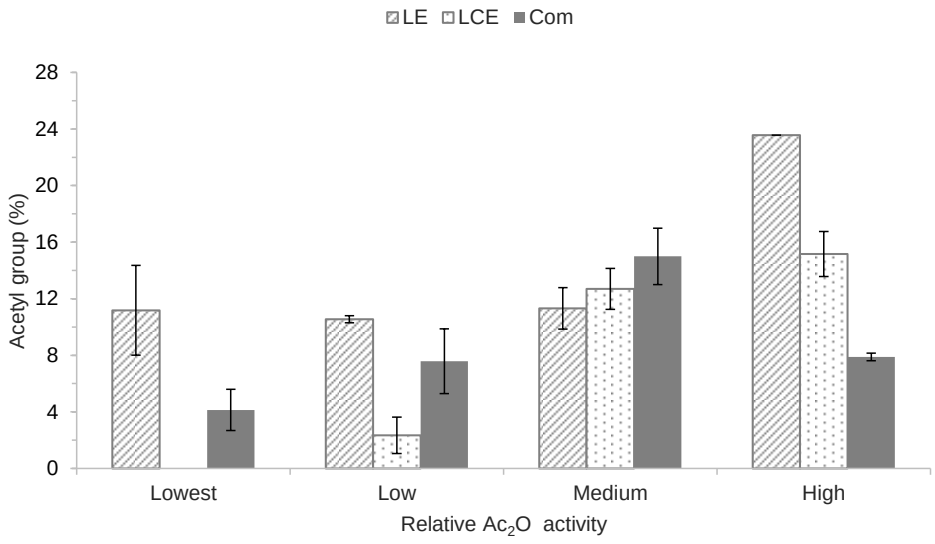


Fig. 4 Content of acetyl group (%) as a function of acetic anhydride used in the respective acetylation reactions for *LE* (diagonal bar), *LCE* (dotted bar) and *Com* (filled bar) samples.

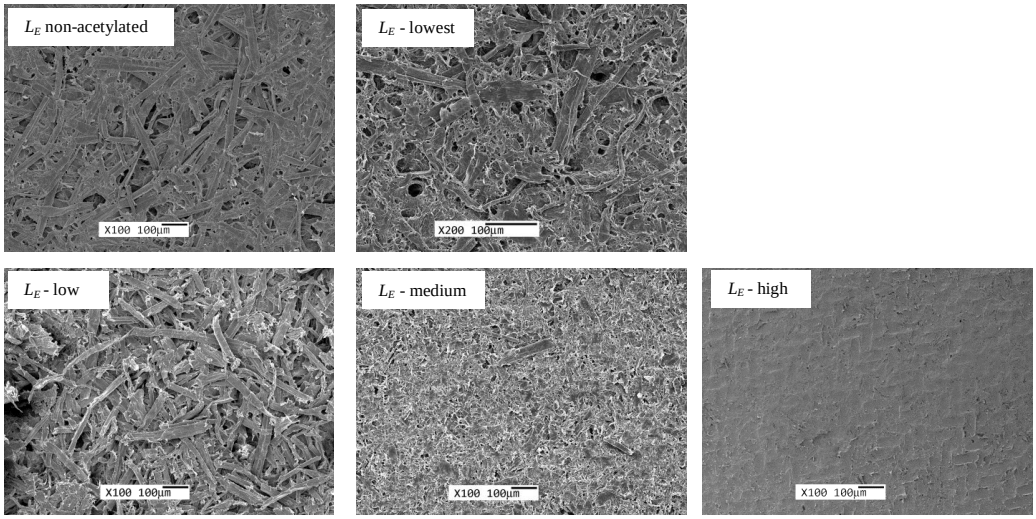
Changes in fiber morphology upon chemo-enzymatic treatment and acetylation

Endoglucanase treatment (L_E) of the biobleached fibers (L) caused a significant reduction (~65%) in fiber length and increase of the fines content (Table S1 of *Supporting Information*). The fiber length decreased from 1.8 mm (L) to 0.68 and 0.62 mm for L_E and L_{CE} samples, respectively. A further length reduction was observed for L_E, L_{CE} and *Com* samples upon acetylation (from lowest to high Ac_2O reaction levels). Specifically, *Com* sample consisted at first of longer fibers than those in L_E and L_{CE}, but at medium and high acetylation conditions (5.35 and 10.7 g Ac_2O) the fiber length reduction and fines generation was more severe for the *Com* sample. A high acetylation degree was achieved in *Com* by using medium level conditions (5.35g Ac_2O). Moreover, fiber length was reduced by about 77% (from 1.33 to 0.30) and fines increased to 65%. However, the greatest reduction in fiber length (87%) and amount of fines generated (> than 90% of fines) took place when high Ac_2O levels were used (10.7 g), indicating the strong degradation of fibers under these conditions. Importantly, the acetyl groups incorporated on the cellulose surface are associated with an increase of mass (coarseness results) and fiber width, and with a reduction in curl. In fact, the strongest effect in these properties was also produced under the "high" conditions of acetylation (coarseness and fiber width increased by 384% and 22% respectively, and fiber curl decreased by 66%). Therefore, the effects on fiber morphology correlate with FTIR results, which indicated an increased acetylation at the "high" conditions. The low values measured for acetyl content in Fig. 4 may be explained by the high fines content measured in the sample.

L_E and L_{CE} also suffered a reduction in fiber length with increasing acetylation degree but, to a lesser extent if compared to the *Com* sample. In particular, at the highest acetylation level (23.6% of acetyl group) of L_E, a mass gain (coarseness) of about 163%, a fiber reduction of about 68% and an increase of fines up to 82% were observed compared to the initial value. Meanwhile, similar values for L_{CE} at the highest acetylation level (15.2% of acetyl groups) were measured (178%, 68% and 84.5%, respectively) (Table S1 of *Supporting Information*).

Surface changes in handsheets of acetylated fibers

The change in the surface morphology of the acetylated fibers was evaluated by scanning electron microscopy (SEM). A clear fiber degradation due to acetylation reactions was confirmed by fiber morphology and also by SEM analyses. As can be seen for all samples treated at the medium Ac_2O level, fiber length was reduced; in addition, the greater amount of fines produced yielded a more entangled structure, with smaller pore size. The greatest changes were observed for fibers subjected to more severe acetylation conditions. In this case, in fact, whole fibers were not observed at the given SEM magnification and the pattern of the mesh used for web preparation was observed (Fig. 5). In addition, the increase in bulk density observed from non-acetylated to the high acetylation conditions indicated a denser and more compact structure (data not shown).



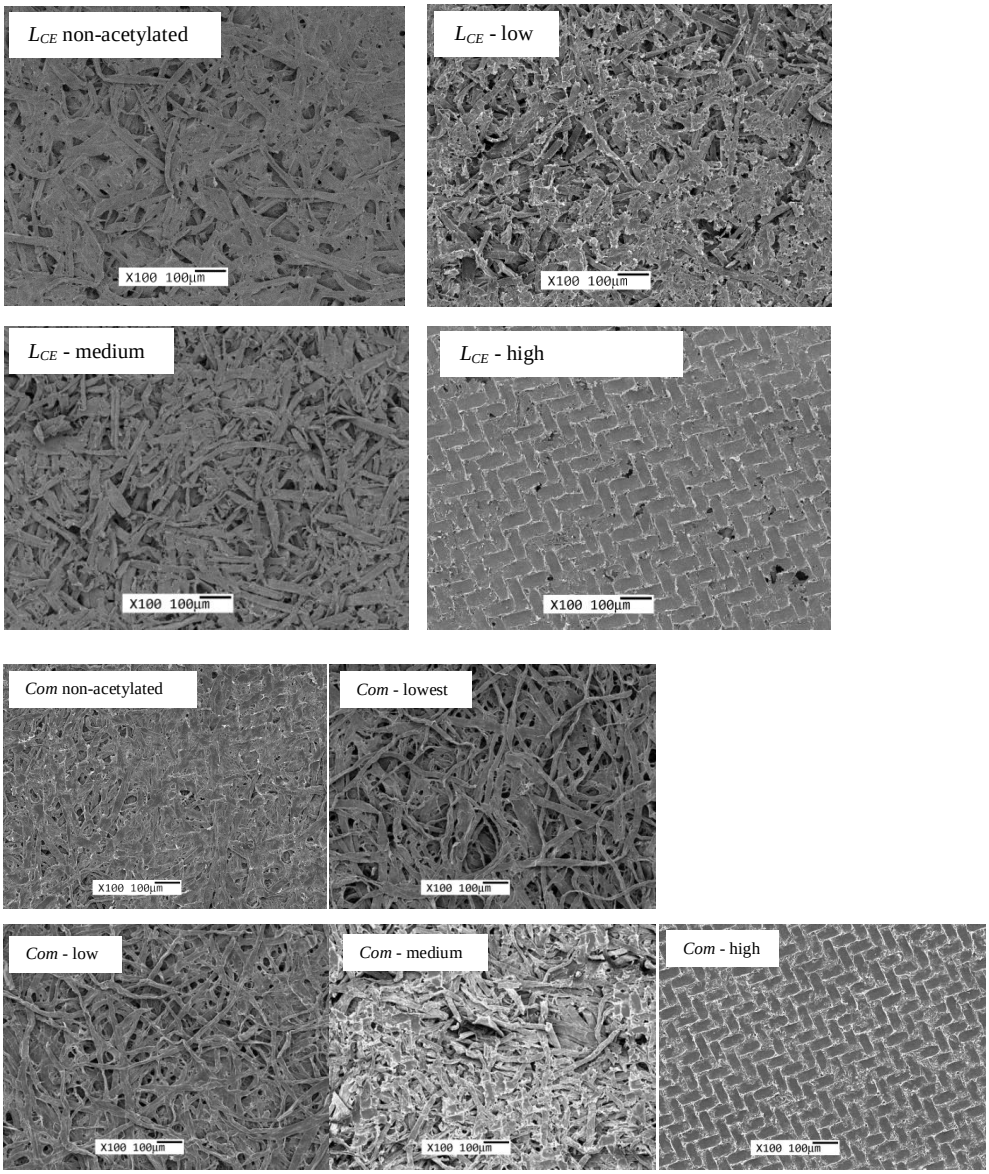


Fig. 5 SEM images of handsheets produced from L_E, L_{CE} and *Com* fibers that were subjected to heterogeneous acetylation at different Ac_2O levels, as indicated

Mechanical properties of the fibers webs

The effect of fiber morphology and acetylation degree was assessed as far as the mechanical properties of the corresponding handsheets (Fig. 6). A high acetylation level is expected to limit hydrogen bonding capacity since acetyl groups substitute –OH's otherwise available for bonding in the cellulose network (Ernest-Saunders et al. 2014). Moreover, the strong deterioration of fibers during acetylation (much shorter and with higher fines content), may yield weaker bonding. Additionally, sheet formation (spatial distribution of mass) was limited

and large variations in the measured physical properties were noted between samples. Generally, a negative effect in tensile strength was observed upon acetylation (Fig. 6a). Fibers subjected to high Ac_2O reaction levels suffered a strength loss of about 70% and 90% for the *L_{CE}* and *Com* fibers, respectively. In addition, the observed strength loss for *L_E* and *L_{CE}* samples correlated with a reduced bulk density. Acetylated fibers from *L_E* and *L_{CE}* samples produced slightly higher wet strength compared to that on non-acetylated fibers (Fig. 6b).

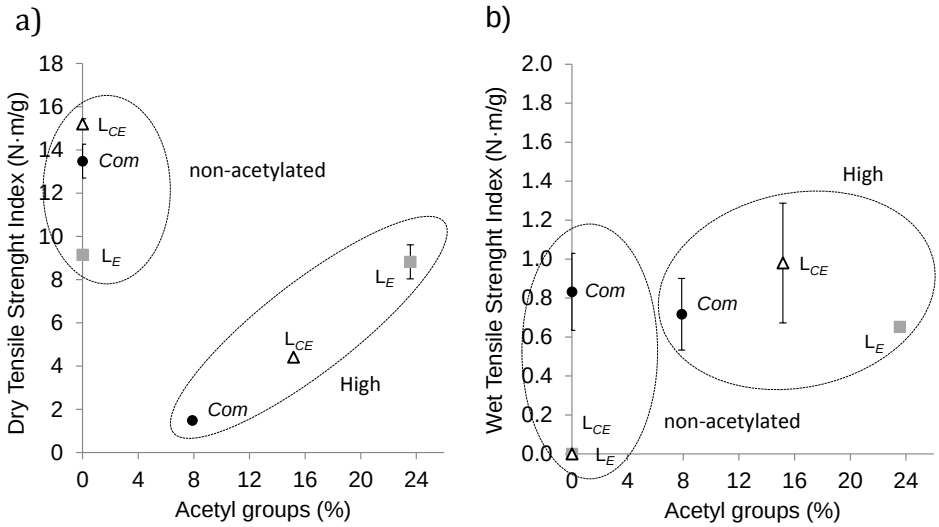


Fig. 6 Dry (a) and wet (b) tensile strength of webs produced with non-acetylated and high acetylated fibers as a function of acetyl group content.

Wetting properties of acetylated fibers

The effect of acetylation treatment on the hydrophilicity of the fibers was examined by means of initial water contact angle (WCA) of the respective handsheets (Figure S2a of *Supporting Information*). In general, the water absorption of paper depends on the porous structure of the sheet and the nature of the interactions that occur between fibers and the fluid (Mashkour et al. 2015). Acetyl groups were expected to reduce the hydrophilicity of fibers and lower the interfiber bonding. The different WCA observed between non-acetylated and acetylated samples confirm the effect of chemical surface modification. Samples with the highest degree of acetylation presented twice the WCA value compared to non-acetylated ones. To be precise, a WCA of 64, 58 and 55° were obtained for acetylated *Com*, *L_E* and *L_{CE}* samples, confirming that the acetylation reactions reduced the hydrophilicity of fibers.

Changes in WCA are mainly due to absorption in the sheet structure and to evaporation—the latter, however, is only relevant for relative long absorption times (Cusola et al. 2013). Water drops were absorbed rapidly for non-acetylated samples, giving an equilibrium WCA close to 0° (Figure S2b of *Supporting Information*). Acetylated L_E and L_{CE} samples also showed fast drop absorption, 2.4 and 56 s respectively (Figure S2c of *Supporting Information*). In contrast, *Com* acetylated sample indicated no change in water drop during 2 min and a WCA of 45º was recorded after 20 min. Finally, after about 36 min the water drop was fully absorbed.

Homogeneous acetylation

Homogeneous acetylation was conducted in order to evaluate the dissolution behavior of fibers treated chemo-enzymatically (L_E and L_{CE}). The results were compared to *Com* reference fibers. In the absence of toluene in the acetylation medium (homogeneous acetylation), a higher percentage of substituted acetyl groups are determined relative to the results from heterogeneous acetylation. FTIR spectroscopy confirmed that acetylation reactions were substantial, as indicated by the fingerprint peak at 1730 cm^{-1} (Fig. 7).

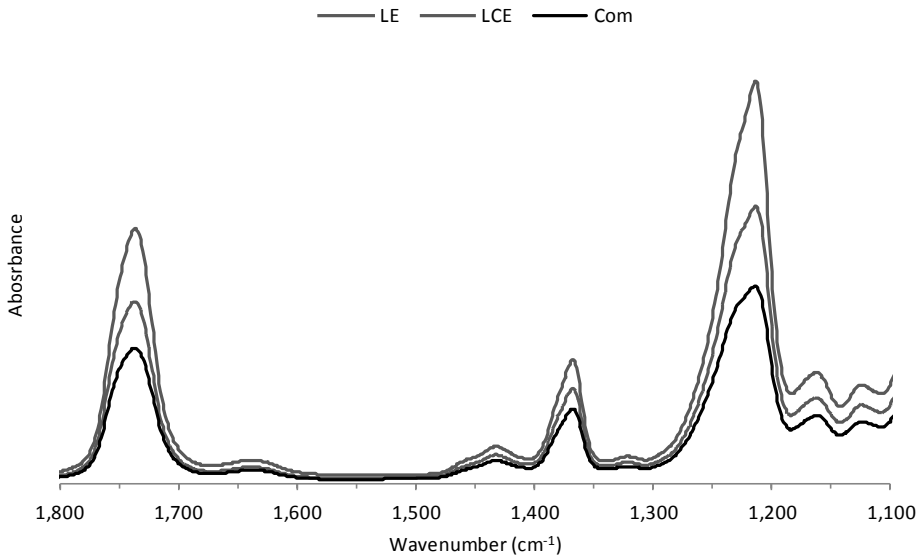


Fig. 7 FTIR spectra for acetylated cellulose from L_E, L_{CE} and *Com* films.

Quantification of the degree of substitution by titration showed similar acetylation degrees for all studied fibers. Values between 33 to 36% of acetyl

substituted groups were found (Table 2), indicating a high level of acetylation comparable to commercial available cellulose acetate (from Sigma-Aldrich ~39%).

Table 2 Acetyl group % determined by the titration method and dry tensile strength index of films produced by solvent casting of L_E, L_{CE} and *Com* samples after homogenous acetylation reaction.

	Acetyl Groups (%)	Dry Tensile Strength Index (N·m/g)	Dry zero-span tensile strength (kN/cm)	Water drop test (s)	Contact Angle (°)
L_E	36.2 ± 4.9	19 ± 3	0.06 ± 0.01	5810 ± 117	76 ± 3
L_{CE}	35.5 ± 3.9	22 ± 11	0.07 ± 0.01	5435 ± 293	67 ± 4
Com	33.3 ± 4.4	67 ± 28	0.05 ± 0.006	5445 ± 507	67 ± 7

Fibers obtained after acetylation were freeze-dried, then dissolved in acetone and the resulting viscous solution was used to prepare films via solvent casting. Films made from the chemo-enzymatic samples presented notably greater strength values than those of heterogeneous acetylation reaction at the highest acetylation level (Table 2). However, despite the fact that a similar acetyl content was measured for all samples, *Com* films presented a tensile strength three times higher than those measured for the samples acetylated after chemo-enzymatic treatment, this can be explained by the differences in fiber morphology of precursor fibers prior to acetylation. In terms of dry zero-span tensile strength, *Com* and chemoenzymatic acetylated fibers showed values in the same range. As observed with heterogeneous acetylation reactions, the presence of acetyl groups reduced the hydrophilic character, giving a contact angle between 67 and 76°. Although high hydrophobicity was not achieved (contact angle < 90°), water drops remained long time on the surface until complete absorption as WDT assay showed. Overall, cellulose acetate fibers with new functional groups and high strength-related properties were achieved.

CONCLUSIONS

Various surface acetylation conditions were studied from a dissolving fiber grade (*Com*) and from a set of newly introduced fibers obtained by chemo-enzymatic treatment of sulfite fibers (L_E, L_{CE}). The respective precursor fibers presented different hemicellulose content, crystallinity and fiber morphology. As

a result, upon given heterogeneous reaction conditions, different acetylation degrees were achieved. FTIR and acetyl group content titrations confirmed the fact that much higher acetyl group content was developed for the more severe acetylation conditions. Morphological studies revealed that acetyl groups were introduced via heterogeneous reactions on the surface of the fibers, as indicated by the gain in coarseness that was observed. Generally, the fiber length decreased with the acetylation degree and a larger amount of fines were produced. Notably, the greatest fiber degradation was observed for *Com* sample under high acetylation conditions giving a 86% fiber length reduction and a gain of about 115% of fines. Handsheets obtained with acetylated fibers exhibited lower dry tensile strength and lower hydrophilicity (determined by contact angle) compared to the non-acetylated grades. Compared to the heterogeneous acetylation, homogeneous reactions led to higher acetyl group degree of substitution. These samples exhibited good solubility in acetone and produced transparent films (via solvent casting) with enhanced dry strength, less hydrophilic character and long time absorption resistance. In conclusion, the synthesis of cellulose esters from the unbleached fibers after the chemo-enzymatic treatment in heterogeneous or homogenous phase (surface or bulk acetylation, respectively) was demonstrated.

ACKNOWLEDGMENTS

The authors thank the ‘‘Ministerio de Economía y Competitividad’’ of Spain for their support in this work under the projects FILMBIOCEL CTQ2016-77936-R (funding also from the ‘‘Fondo Europeo de Desarrollo Regional FEDER’’) and MICROBIOCEL CTQ2017-84966-C2-1-R. The authors are grateful to the Serra Húnter Fellow to Cristina Valls. The authors would like to thank DOMSJÖ (Sweden) for providing the starting pulp. O.J.R. acknowledges funding support by the Academy of Finland through its Center of Excellence Program (2014-2019) “Molecular Engineering of Biosynthetic Hybrid Materials Research” (HYBER).

REFERENCES

Ass BAP, Frollini E, Heinze T (2004) Studies on the homogeneous acetylation of cellulose in the novel solvent dimethyl sulfoxide/tetrabutylammonium fluoride trihydrate. Macromol Biosci 4:1008–1013. doi: 10.1002/mabi.200400088

Barud HS, de Araújo Júnior AM, Santos DB, et al (2008) Thermal behavior of cellulose acetate produced from homogeneous acetylation of bacterial cellulose. Thermochim Acta 471:61–69. doi: 10.1016/j.tca.2008.02.009

Cao Y, Wu J, Meng T, et al (2007) Acetone-soluble cellulose acetates prepared by one-step homogeneous acetylation of cornhusk cellulose in an ionic liquid 1-allyl-3-methylimidazolium chloride (AmimCl). Carbohydr Polym 69:665–672. doi: 10.1016/j.carbpol.2007.02.001

Cunha AG, Zhou Q, Larsson PT, Berglund LA (2014) Topochemical acetylation of cellulose nanopaper structures for biocomposites: mechanisms for reduced water vapour sorption. Cellulose 21:2773–2787. doi: 10.1007/s10570-014-0334-z

Cusola O, Valls C, Vidal T, Roncero MB (2013) Application of surface enzyme treatments using laccase and a hydrophobic compound to paper-based media. Bioresour Technol 131:521–6. doi: 10.1016/j.biortech.2012.12.186

Engström A-C, Ek M, Henriksson G (2006) Improved Accessibility and Reactivity of Dissolving Pulp for the Viscose Process: Pretreatment with Monocomponent Endoglucanase. Biomacromolecules 7:2027–2031.

Ernest-Saunders R, Pawlak JJ, Lee JM (2014) Properties of surface acetylated microfibrillated cellulose relative to intra- and inter-fibril bonding. Cellulose 21:1541–1552. doi: 10.1007/s10570-014-0177-7

FAO (2012) FAO. Food and Agriculture Organization of the United Nations.

Fischer S, Thümmler K, Volkert B, et al (2008) Properties and applications of cellulose acetate. Macromol Symp 262:89–96. doi: 10.1002/masy.200850210

Fock W (1959) A modified method for determining the reactivity of viscose-grade dissolving pulps. Papier 13:92–95.

Gehmayr V, Sixta H (2011) Dissolving pulp from enzyme treated kraft pulps for viscose application. Lenzinger berichte 89:152–160.

Janzon R, Puls J, Bohn A, et al (2008a) Upgrading of paper grade pulps to dissolving pulps by nitren extraction: yields, molecular and supramolecular structures of nitren extracted pulps. Cellulose 15:739–750. doi: 10.1007/s10570-008-9224-6

Janzon R, Saake B, Puls J (2008b) Upgrading of paper-grade pulps to dissolving pulps by nitren extraction: Properties of nitren extracted xylans in comparison to NaOH and KOH extracted xylans. Cellulose 15:161–175. doi: 10.1007/s10570-007-9154-8

621 Jegannathan KR, Nielsen PH (2013) Environmental assessment of enzyme use in industrial
622 production-a literature review. J Clean Prod 42:228–240 . doi: 10.1016/j.jclepro.2012.11.005

623 Kalia S, Boufi S, Celli A, Kango S (2014) Nanofibrillated cellulose: Surface modification and
624 potential applications. Colloid Polym Sci 292:5–31. doi: 10.1007/s00396-013-3112-9

625 Köpcke V, Ibarra D, Ek M (2008) Increasing accessibility and reactivity of paper grade pulp by
626 enzymatic treatment for use as dissolving pulp. Nord Pulp Pap Res J 23:363–368.

627 Köpcke V, Ibarra D, Larsson PT, Ek M (2010) Optimization of treatment sequences for the
628 production of dissolving pulp from birch kraft pulp. Nord Pulp Pap Res J 25:31–38.

629 Krässig HA (1993) Cellulose-Structure, Accessibility and Reactivity. Gordon and Breach Science
630 Publisher, Yverdon, Switzerland and Philadelphia, p 376

631 LaNieve HL, Richard K (2007) Handbook of fiber chemistry. In: Lewin M (ed) Cellulose acetate
632 and triacetate fibers, 3rd editio. pp 667–772

633 Luo P, Cao C, Liang Y, et al (2013) Kinetic Study of the Acetylation of Cotton Linter Pulp.
634 BioResources 8:2708–2718. doi: 10.15376/biores.8.2.2708-2718

635 Mashkour M, Afra E, Resalati H, Mashkour M (2015) Moderate surface acetylation of
636 nanofibrillated cellulose for the improvement of paper strength and barrier properties. RSC Adv
637 5:60179–60187. doi: 10.1039/c5ra08161k

638 Muhammad Djuned F, Asad M, Mohamad Ibrahim MN, Wan Daud WR (2014) Synthesis and
639 Characterization of Cellulose Acetate from TCF Oil Palm Empty Fruit Bunch Pulp. BioResources
640 9:4710–4721. doi: 10.15376/biores.9.3.4710-4721

641 Quintana E, Valls C, Vidal T, Roncero MB (2015a) Comparative evaluation of the action of two
642 different endoglucanases. Part II: On a biobleached acid sulphite pulp. Cellulose 22:2081–2093.
643 doi: 10.1007/s10570-015-0631-1

644 Quintana E, Valls C, Vidal T, Roncero MB (2015b) Comparative evaluation of the action of two
645 different endoglucanases. Part I: On a fully bleached, commercial acid sulfite dissolving pulp.
646 Cellulose 22:2067–2079. doi: 10.1007/s10570-015-0623-1

647 Quintana E, Valls C, Vidal T, Roncero MB (2013) An enzyme-catalysed bleaching treatment to
648 meet dissolving pulp characteristics for cellulose derivatives applications. Bioresour Technol
649 148:1–8. doi: 10.1016/j.biortech.2013.08.104

650 Rodionova G, Lenes M, Eriksen Ø, Gregersen Ø (2011) Surface chemical modification of
651 microfibrillated cellulose: Improvement of barrier properties for packaging applications. Cellulose
652 18:127–134. doi: 10.1007/s10570-010-9474-y

653 Roselli A, Hummel M, Monshizadeh A, et al (2014) Ionic liquid extraction method for upgrading
654 eucalyptus kraft pulp to high purity dissolving pulp. Cellulose 21:3655–3666. doi:
655 10.1007/s10570-014-0344-x

Saka S, Matsumura H (2004) 2.3 Wood pulp manufacturing and quality characteristics. Macromol Symp 208:37–48. doi: 10.1002/masy.200450404

Skals PB, Krabek A, Nielsen PH, Wenzel H (2008) Environmental assessment of enzyme assisted processing in pulp and paper industry. Int J Life Cycle Assess 13:124–132 . doi: 10.1065/lca2007.11.366

Steinmeier H (2004) Chemistry of cellulose acetylation. Macromol Symp 208:49–60. doi: 10.1002/masy.200450405

Wan Daud WR, Djuned FM (2015) Cellulose acetate from oil palm empty fruit bunch via a one step heterogeneous acetylation. Carbohydr Polym 132:252–260. doi: 10.1016/j.carbpol.2015.06.011

Zhi Fu G, Chan A, Minns D (2005) Preliminary Assessment of the Environmental Benefits of Enzyme Bleaching for Pulp and Paper Making (7 pp). Int J Life Cycle Assess 10:136–142 . doi: 10.1065/lca2004.06.162

Figures

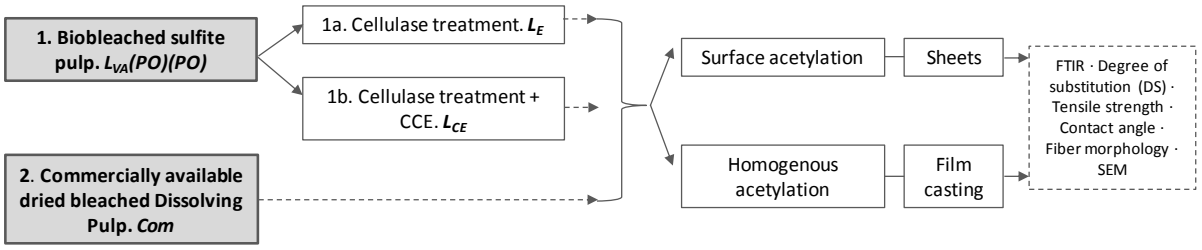


Fig. 1

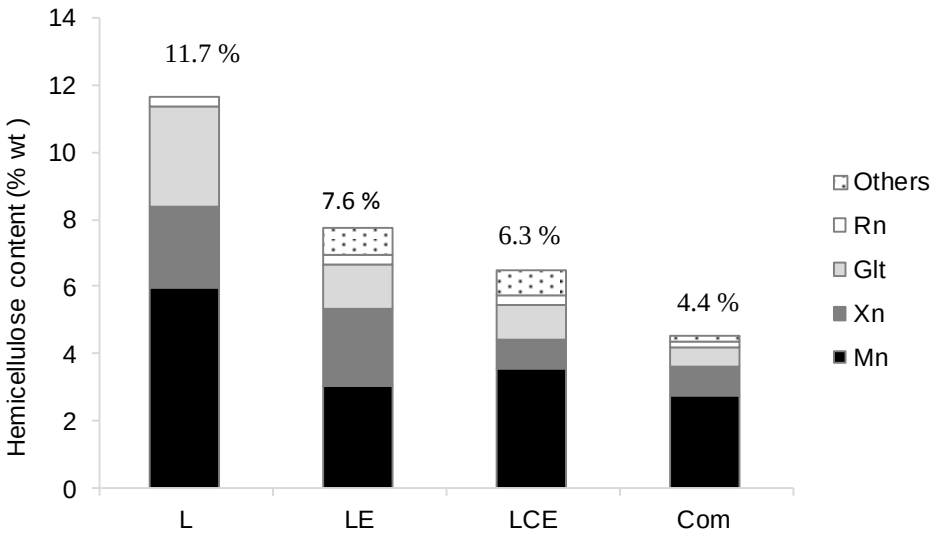

14
12
10
8
6
4
2
0
Hemicellulose content (% wt)
11.7 %
7.6 %
6.3 %
4.4 %
L
LE
LCE
Com
Others
Rn
Glt
Xn
Mn

Fig. 2

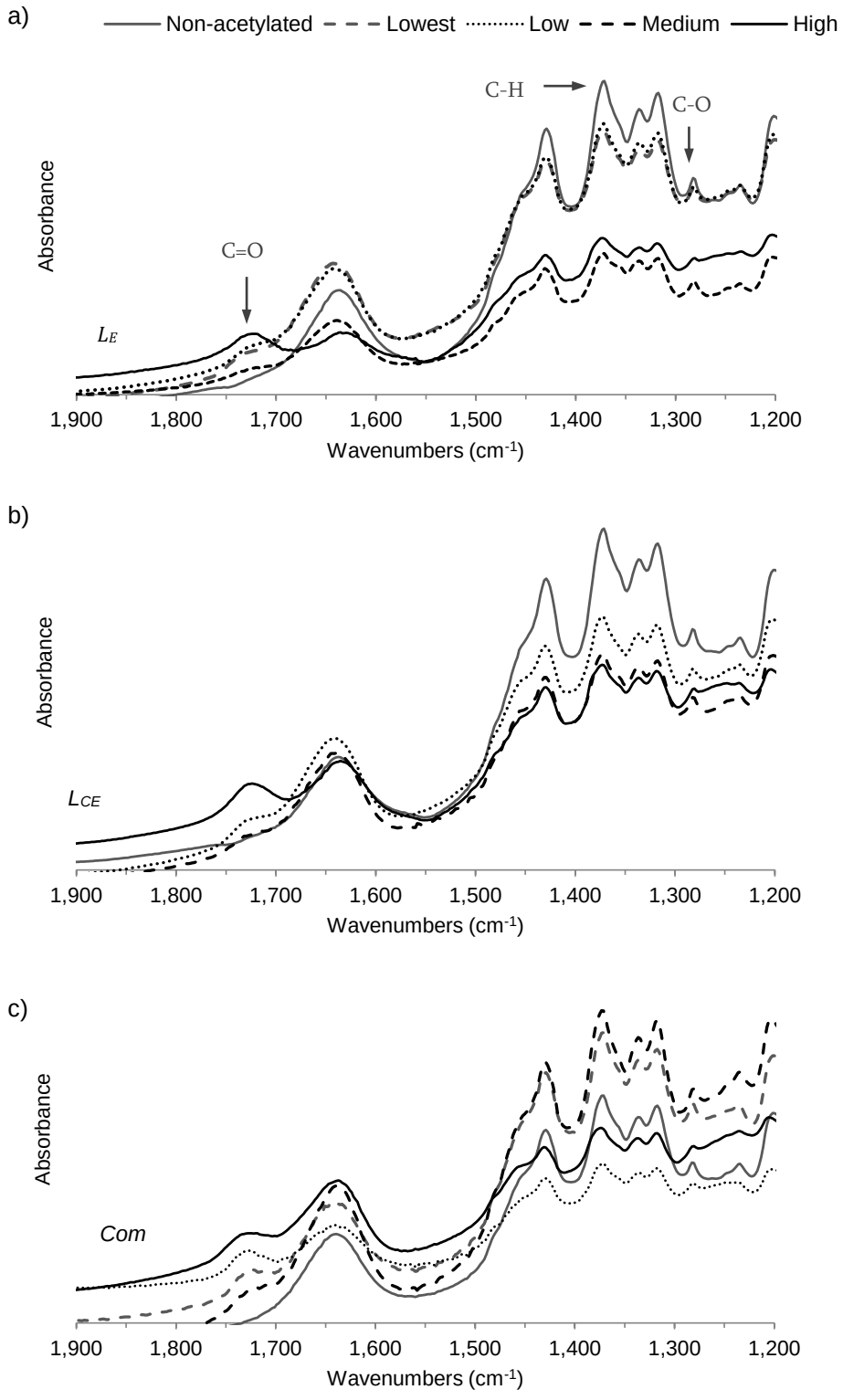

a)
Non-acetylated
Lowest
Low
Medium
High
C-H
C-O
C=O
Absorbance
LE
1,900
1,800
1,700
1,600
1,500
1,400
1,300
1,200
Wavenumbers (cm-1)
b)
Absorbance
LCE
1,900
1,800
1,700
1,600
1,500
1,400
1,300
1,200
Wavenumbers (cm-1)
c)
Absorbance
Com
1,900
1,800
1,700
1,600
1,500
1,400
1,300
1,200
Wavenumbers (cm-1)

Fig. 3

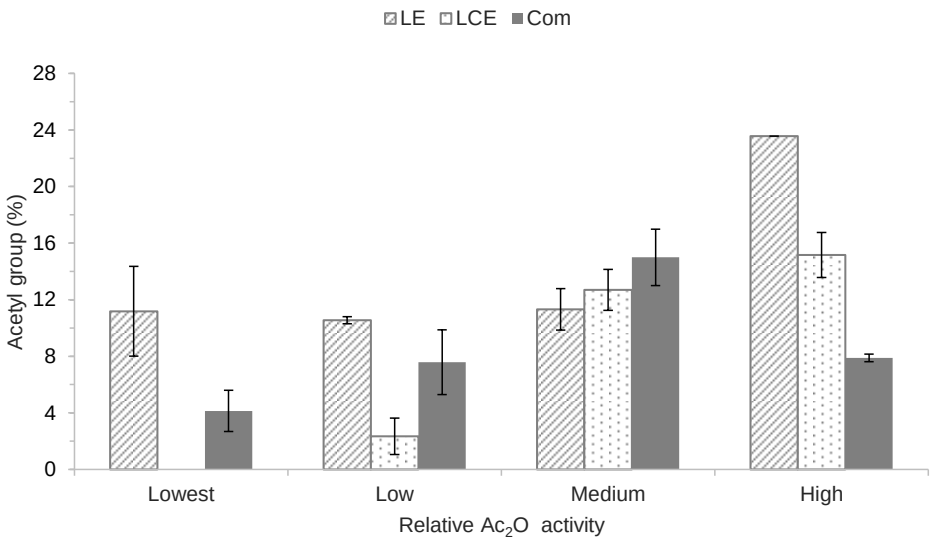
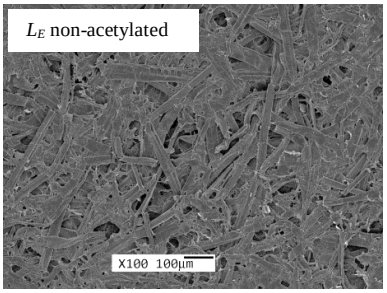
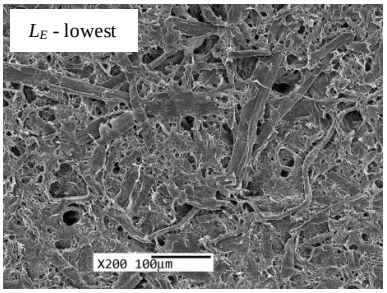
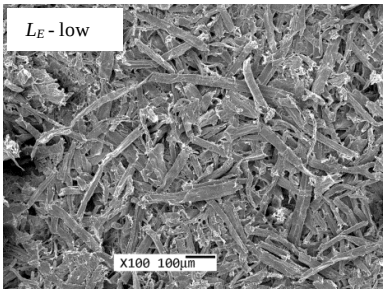
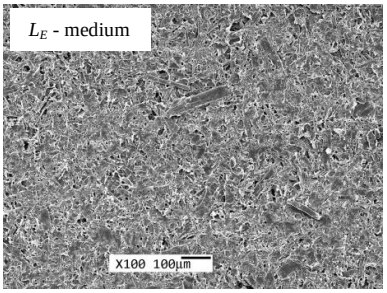

LE
LCE
Com
28
24
20
16
12
8
4
0
Acetyl group (%)
Lowest
Low
Medium
High
Relative Ac_2O activity

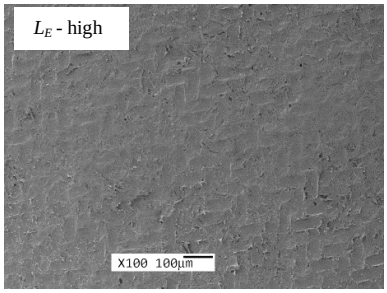
Fig. 4

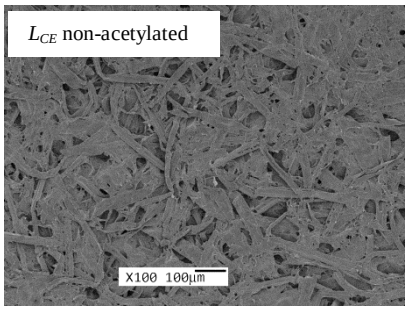

L_E non-acetylated
X100 100µm

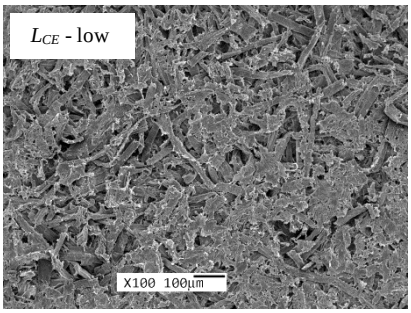

L_E - lowest
X200 100µm

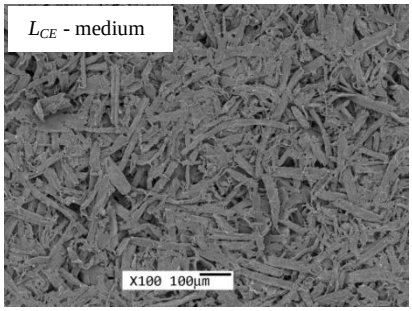

L_E - low
X100 100µm

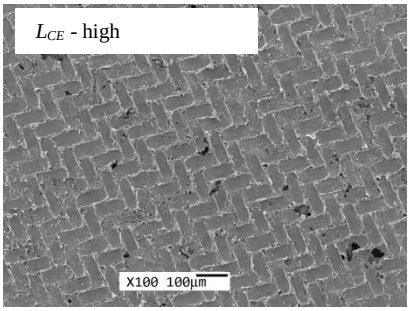

L_E - medium
X100 100µm


L_E - high
X100 100µm


L_{CE} non-acetylated
X100 100µm


L_{CE} - low
X100 100µm


L_{CE} - medium
X100 100µm


L_{CE} - high
X100 100µm

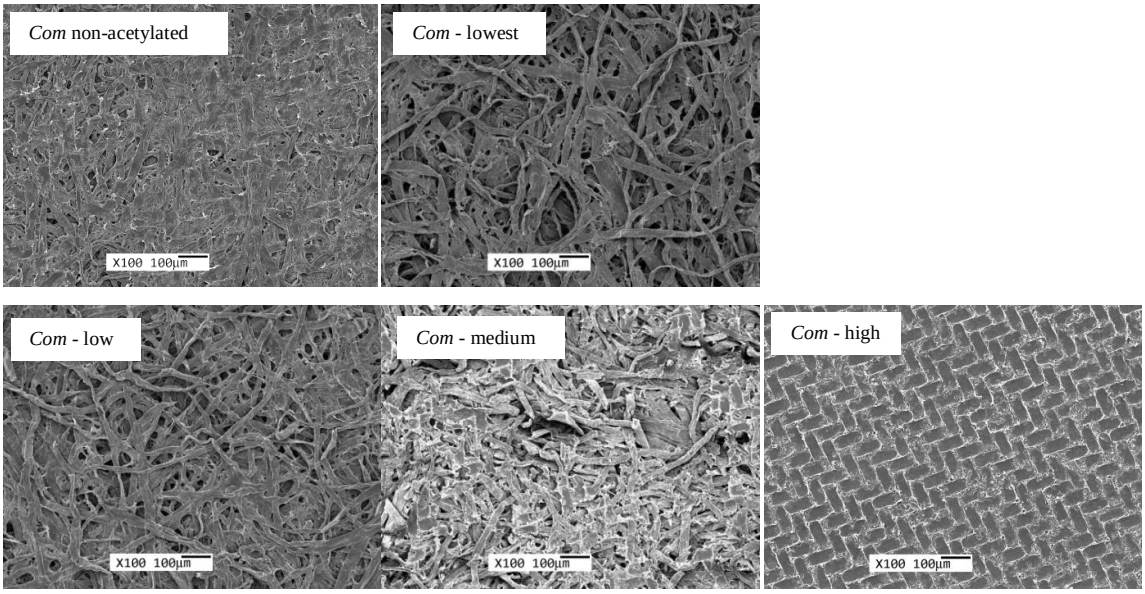

Com non-acetylated
X100 100µm
Com - lowest
X100 100µm
Com - low
X100 100µm
Com - medium
X100 100µm
Com - high
X100 100µm

Fig. 5

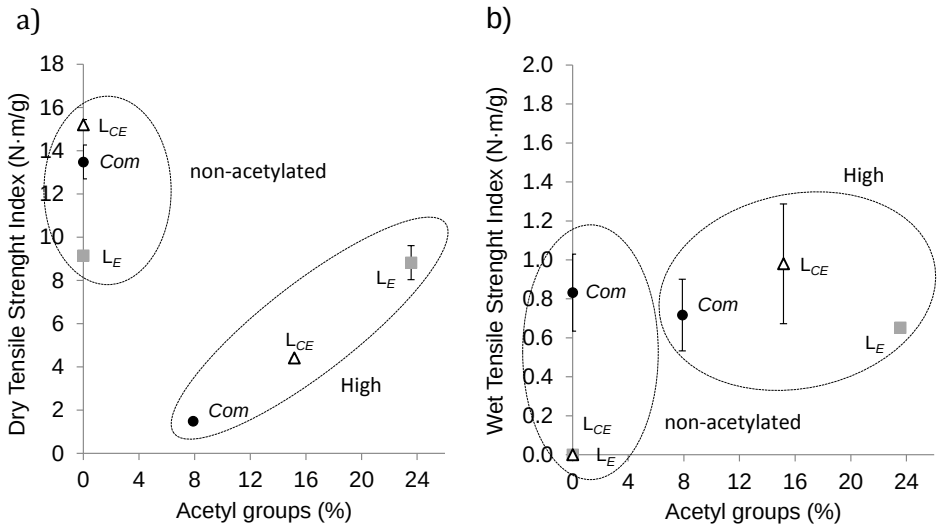

a)
b)
Dry Tensile Strenght Index (N·m/g)
Wet Tensile Strenght Index (N·m/g)
Acetyl groups (%)
Acetyl groups (%)
non-acetylated
High
Com
L_{CE}
L_E
non-acetylated
High

Fig. 6

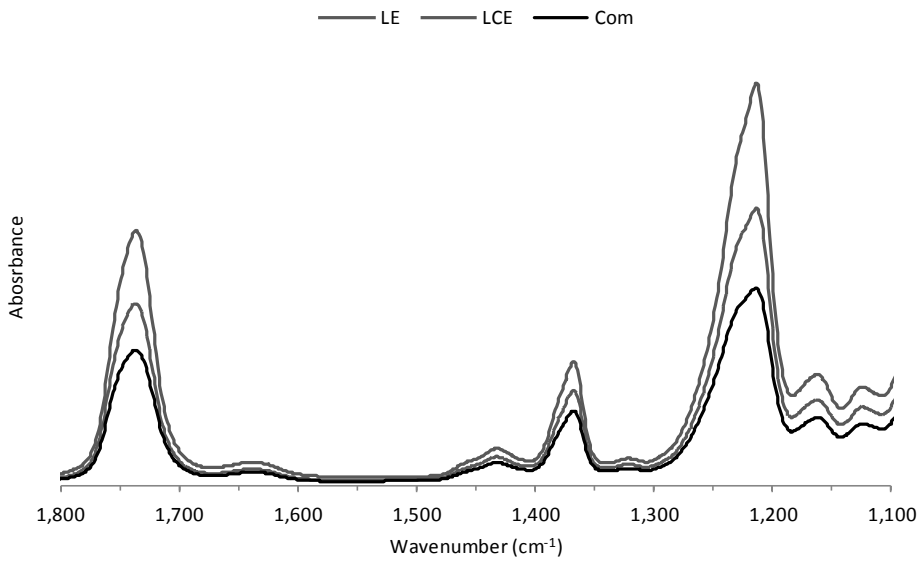

LE
LCE
Com
Abosrbance
1,800
1,700
1,600
1,500
1,400
1,300
1,200
1,100
Wavenumber (cm-1)

Fig. 7